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Gibberellin biosynthesis and gibberellin oxidase activities in *Fusarium sacchari*, *Fusarium konzum* and *Fusarium subglutinans* strains

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ABSTRACT

Several isolates of three *Fusarium* species associated with the *Gibberella fujikuroi* species complex were characterized for their ability to synthesize gibberellins (GAs): *Fusarium sacchari* (mating population B), *Fusarium konzum* (mating population I) and *Fusarium subglutinans* (mating population E). Of these, *F. sacchari* is phylogenetically related to *Fusarium fujikuroi* and is grouped in the Asian clade of the complex, while *F. konzum* and *F. subglutinans* are only distantly related to *Fusarium fujikuroi* and belong to the American clade. Variability was found between the different *F. sacchari* strains tested. Five isolates (B-12756; B-1732, B-7610, B-1721 and B-1797) were active in GA biosynthesis and accumulated GA_3 in the culture fluid (2.76–28.4 μg/mL), while two others (B-3828 and B-1725) were inactive. GA_3 levels in strain B-12756 increased by 2.9 times upon complementation with *ggs2* and *cps-ks* genes from *F. fujikuroi.* Of six *F. konzum* isolates tested, three (I-10653; I-11616; I-11893) synthesized GAs, mainly GA_1, at a low level (less than 0.1 μg/mL). Non-producing *F. konzum* strains contained no GA oxidase activities as found for the two *F. subglutinans* strains tested. These results indicate that the ability to produce GAs is present in other species of the *G. fujikuroi* complex beside *F. fujikuroi*, but might differ significantly in different isolates of the same species.

1. Introduction

The *Gibberella fujikuroi* species complex consists of eleven sexually fertile species (also known as mating populations, MPs) and at least 32 anamorphs mostly corresponding to the genus *Fusarium* section Liseola (Leslie and Summerell, 2006). Recently, MPs have been assigned *Gibberella* species names parallel to the names of their anamorphs (O'Donnell et al., 2000; Zeller et al., 2003). These *Fusarium* species have been isolated from a variety of host plants including maize, rice, pine, banana, coffee and sugar cane (Leslie, 1995, 1999). The fungal species produce a range of secondary metabolites such as either mycotoxins (Marasas et al., 1984; Nelson et al., 1992; Leslie, 1995) or the plant hormones, gibberellins (GAs) (Leslie, 1995; Desjardins, 2003; Malonek et al., 2005a). Production of GAs has been described so far mainly in *Fusarium fujikuroi*, the causative agent of the bakanae disease of rice that synthesizes high levels of gibberellic acid (GA_3) (**19**) (see Fig. 1), which causes the characteristic overgrowth symptoms in infected plants. All *F. fujikuroi* strains so far analyzed produce GAs although with differences in yield and composition (Malonek et al., 2005a). Besides *F. fujikuroi,* only particular strains of two other species of the complex, *Fusarium* konzum and *Fusarium proliferatum*, have been found to produce GAs. *F. konzum* strain I-10653, isolated from prairie grass (Leslie et al., 2004), synthesizes GA_1 (**18**) and expresses the GA genes, in contrast to other *F. konzum* isolates that neither produce GAs nor express GA genes (Malonek et al., 2005a). Additionally one *F. proliferatum* strain recently isolated from the roots of an orchid, produces mainly GA_4 (**15**) and GA_7 (**17**) (Tsavkelova et al., 2008), while *F. proliferatum* strain KGL0401, isolated from the roots of *Physalis alkekengi*, synthesizes a broad range of GAs, including GA_3 (**19**) (Rim et al., 2005). In contrast, other *F. proliferatum* strains do not produce GAs (Malonek et al., 2005a,b,c). The non-producing strains contain all the GA-biosynthetic genes, but their expression levels are low. In addition, many mutations have accumulated in the coding and 5′-non-coding regions of several of the genes. In strain D00502, the three early steps of GA biosynthesis, catalyzed by GGS2, CPS-KS and P450-4 (Fig. 1) are blocked, in contrast to steps mediated by GA_{14} synthase (P450-1), GA 20-oxidase (P450-2) and 13-hydroxylase (P450-3) that are active (Malonek et al., 2005b,c). Interestingly, strain D02945 lacks GA_{14} synthase activity, which demonstrates the genetic diversity within this species (Malonek et al., 2005b). For other GA-non-producing species of the *G. fujikuroi* complex, the pathway

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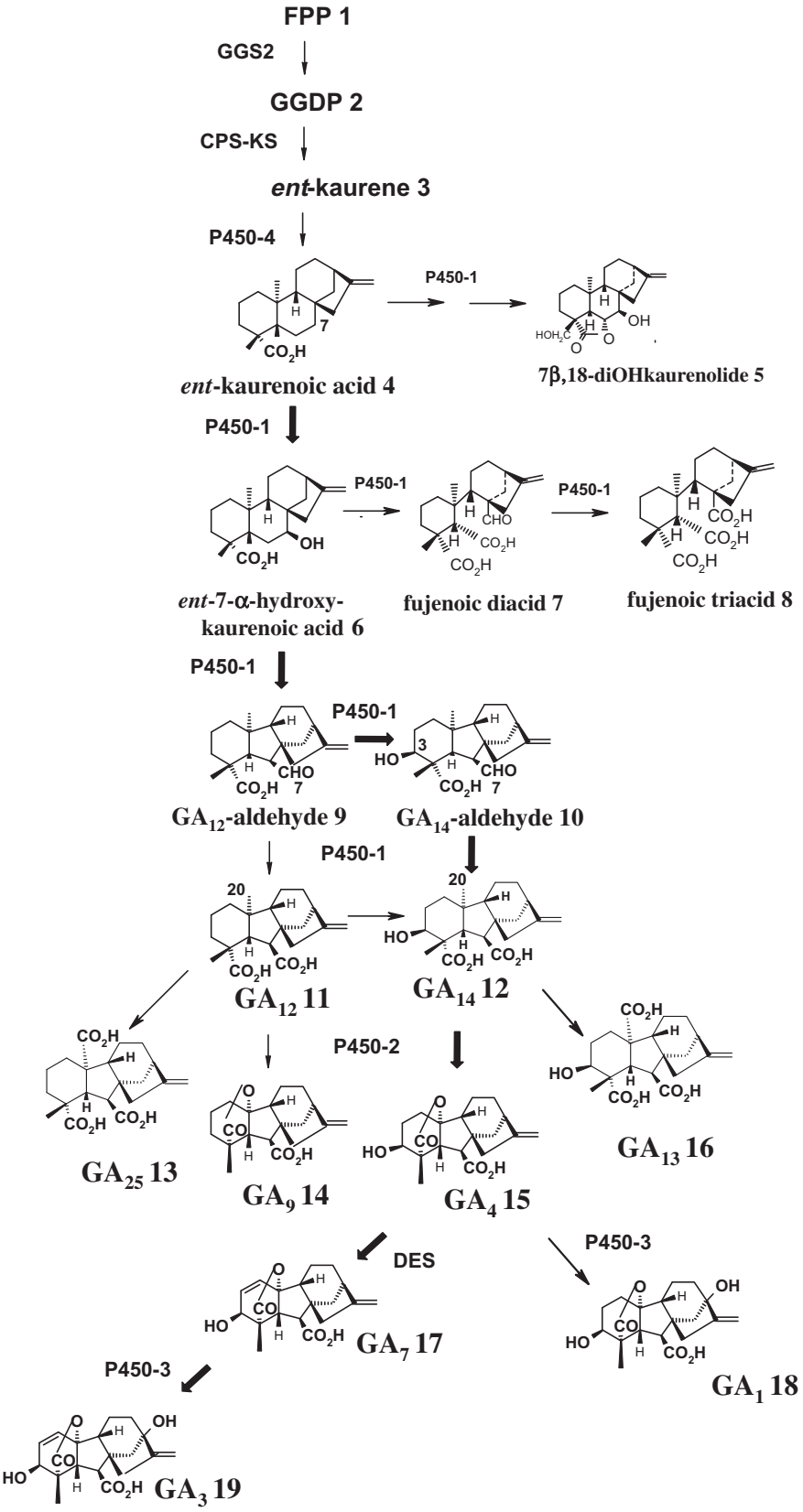

Fig. 1. Kaurenoid and GA metabolism in the *G. fujikuroi* species complex. Thick arrows indicate the proposed major pathway from *ent*-kaurenoic acid in *F. fujikuroi*.

could also be blocked at early steps, as in *F. proliferatum,* but this has not been demonstrated.

Different isolates of the other species of the complex so far studied do not produce GAs although many contain the entire gene cluster (Malonek et al., 2005a). Exceptions are *Fusarium circinatum* and *Fusarium* verticillioides for which only one and two GA genes, respectively, remain in the genomes (Malonek et al., 2005a; Bömke et al., 2008).

Phylogenetic analysis of *Fusarium* species based on the sequences of the ITS regions of ribosomal DNA, the mitochondrial small subunit (mtSSU) rDNA, or protein-encoding marker genes such as β-tubulin, translation elongation factor (TEF) or histone genes, demonstrated that the species belonging to the *G. fujikuroi* complex are closely related and have a monophyletic origin (O'Donnell and Cigelnik, 1997; O'Donnell et al., 2000; Proctor et al., 2004). Based on these analyses, the members of the complex have been delineated into three lineages, designated as the African, Asian and American clades (O'Donnell et al., 1998, 2000). Phylogenetic analysis based on sequence comparison of the GA-biosynthetic gene *P450-4* and of the cytochrome P450 reductase gene *cpr-Gf*, support this classification (Malonek et al., 2005a). The *Fusarium* GA-producing species described so far belong to different lineages: *F. fujikuroi* and *F. proliferatum* belong to the Asian clade, while *F. konzum* is distantly related to *F. fujikuroi* and is classified in the American clade (Malonek et al., 2005a).

Detailed characterization of GA biosynthesis has been described in *F. fujikuroi* at the chemical, enzymatic, and genetic levels (reviewed in Hedden et al., 2002; Tudzynski, 2005; Bömke and Tudzynski, 2009). The pathway consists of two early cyclization reactions, from geranylgeranyl diphosphate (GGDP) to *ent*-kaurene via *ent*-copalyl diphosphate (CDP), followed by multiple oxidative steps to give the final 19–10 γ-lactone product, GA_3 (**19**) (Fig. 1). As for many fungal secondary metabolic pathways, in *F. fujikuroi* the seven GA-biosynthetic genes are organized in a cluster (Tudzynski and Hölter, 1998; Linnemannstöns et al., 1999). They include four cytochrome P450 monooxygenase genes (*P450-1* to *P450-4*), a pathway-specific GGDP synthase gene (*ggs2*), an *ent*-copalyl diphosphate/*ent*-kaurene synthase gene (*cps/ks*) and a GA_4 desaturase gene (*des*) (Tudzynski et al., 1998; Rojas et al., 2001; Tudzynski et al., 2001, 2002, 2003). The P450 GA monooxygenases are multifunctional and catalyze most of the reactions of the pathway (Fig. 1): P450-4 (*ent*-kaurene oxidase) catalyzes the three-step oxidation of *ent*-kaurene (**3**) to *ent*-kaurenoic acid (**4**) (Tudzynski et al., 2001), P450-1 (GA_{14} synthase) converts *ent*-kaurenoic acid (**4**) to GA_{14} (**12**) in four steps including C-7 oxidation and 3β-hydroxylation (Rojas et al., 2001) as well as catalyzing the side-reactions to kaurenolides and fujenoic acids (Rojas et al., 2004), while P450-2 (GA 20-oxidase) catalyzes the removal of C-20 as CO_2 to give the C_{19}-GA skeleton (Tudzynski et al., 2002). The fourth monooxygenase, P450-3, catalyzes hydroxylation on C-13 of GA_4 (**15**) or GA_7 (**17**) in the last steps of the pathway (Tudzynski et al., 2003). The fungal GA monooxygenases obtain electrons from NADPH through the cytochrome P450 reductase encoded by *Gf-cpr* (Malonek et al., 2004). Except for P450-3, expression of the GA-biosynthetic genes is regulated by nitrogen metabolite repression mediated by the transcription factor AreA (Tudzynski et al., 1999; Mihlan et al., 2003; Schönig et al., 2008).

In this work, we investigated the ability to produce GAs and describe the functional analysis of GA oxidases in three species of the *G. fujikuroi* complex that contain the entire GA gene cluster: *Fusarium* sacchari isolated from sugar cane (Leslie, 1995), *F. konzum* isolated from prairie grass (Zeller et al., 2003) and *Fusarium* subglutinans isolated from maize (Desjardins et al., 2000). *F. sacchari* (MP-B) is closely related to *F. fujikuroi* and belongs to the Asian clade, while *F. konzum* (MP-I) and *F. subglutinans* (MP-E) are distantly related to *F. fujikuroi* and belong to the American clade (Malonek et al., 2005a). Previously, genetic diversity has been described in *F. konzum* since one GA-producing and several non-producing strains have been found (Malonek et al., 2005a). On the other hand, no C_{19}-GAs were detected by HPLC analysis in the culture fluid of several *F. subglutinans* and *F. sacchari* strains grown under nitrogen-limiting conditions (Malonek et al., 2005a). Here we investigate GA biosynthesis, by GC–MS analysis as well as by administrating ^{14}C-labelled precursors, in more isolates of these three *Fusarium* species and identify GA-producing and GA-non-producing strains in *F. sacchari* and in *F. konzum*, while none of the *F. subglutinans* strains tested synthesize GAs. Our results contribute to the understanding of the distribution of GA biosynthesis within the *G. fujikuroi* species complex and in fungal systems in general.

2. Results

2.1. Gibberellins and ent-kaurenoids present in F. sacchari (MP-B) cultures

GA biosynthesis was investigated in seven *F. sacchari* strains grown for 10 days in a chemically defined liquid media containing limiting nitrogen (10% ICI). In addition to strain B-1725 that had been previously tested (Malonek et al., 2005a), we investigated six other isolates including strain B-7610 already reported as a GA-producer (Kawaide, 2006). GC–MS analysis of culture filtrate extracts established the presence of GA-producing and GA-non-producing strains (Table 1, Fig. 2). Five strains, B-1732, B-12756, B-7610, B-1721 and B-1797, synthesised GAs, mainly GA_3 (**19**) in contrast to the cultures of strains B-3828 and B-1725 that possessed very low GA-biosynthetic activity, producing only traces of GA_3 (**19**) and/or GA_1 (**18**). Particularly strains B-12756 and B-1732 accumulated substantial amounts of GA_3 (**19**) after 10 days in culture (28.4 and 27.2 μg/mL, respectively; Table 2) while the cultures of B-7610 and B-1721 contained lower amounts of this final product, 11.36 and 2.76 μg/mL, respectively (Table 2). Only 3β-hydroxylated GAs were found in the cultures of B-1732, B-7610 and B-1797, while low levels of non-hydroxylated C_{19}- and C_{20}-GAs were found in strains B-12756 and B-1721 in addition to 3β-hydroxylated GAs (Table 1). This indicates that, as in *F. fujikuroi*, the 3β-hydroxylation pathway is the main pathway of GA biosynthesis in *F. sacchari*. Gibberellenic acid, iso-GA_3 and GA_4 diacid 1,10-ene detected in some extracts are probably non-enzymatic products formed during either the incubation or extraction at low pH, while GA_{47} (**25**) (2α-hydroxyGA_4), GA_{16}

Table 1
GAs and *ent*-kaurenoids identified by GC–MS in the culture fluid of *F. sacchari* (MP-B) strains.

Strain	Gibberellins	*ent*-Kaurenoids	GA_3:GA_1
B-1732	GA_4, **15**; gibberellenic acid; GA_{13}, **16**; GA_{36}, **22**; iso-GA_3; GA_1, **18**; GA_3, **19** (strong)	Fujenoic diacid, **7** Fujenoic triacid, **8** 7β,18-diOHkaurenolide, **5**	1:0.55
B-12756	GA_9, **14**; GA_{24}, **21**; GA_{25}, **13**; GA_{14}, **12**; GA_4, **15**; GA_7, **17**; gibberellenic acid; GA_{13}, **16**; GA_{36}, **22**; GA_{47}, **25**; iso-GA_3; GA_{16}, **23**; GA_1, **18**; GA_3, **19**	Fujenoic diacid, **7** Fujenoic triacid, **8** 7β,18-diOHkaurenolide,**5**	1:0.17
B-7610	GA_4,**15**; GA_7,**17**; GA_{13},**16** (trace); GA_1,**18** (trace); GA_3,**19**	Fujenoic diacid, **7**	1:0.14
B-1721	GA_9, **14**; GA_4 diacid 1,10-ene; GA_{25}, **13**; GA_{24}, **21**; GA_4, **15**; gibberellenic acid; GA_{13}, **16**; GA_{36}, **22**; GA_1, **18**; GA_3, **19**; 2-epi-GA_{43} (strong)	7β-OHkaurenolide Fujenoic diacid, **7** Fujenoic triacid, **8** 7β,18-diOHkaurenolide, **5**	1:0.37
B-1797	GA_{14}, **12**; GA_4, **15**; gibberellenic acid; GA_{13}, **16**; GA_{36}, **22**; GA_{47}, **25**; iso-GA_3; GA_{16}, **23**; GA_1, **18**; GA_3, **19**	Fujenoic diacid, **7** Fujenoic triácido, **8** 7β,18-diOHkaurenolide, **5**	1:0.32
B-3828	GA_3, **19** (trace)	–	–
B-1725	GA_3, **19** (trace); GA_1, **18** (trace)	Fujenoic diacid, **7** (trace)	–

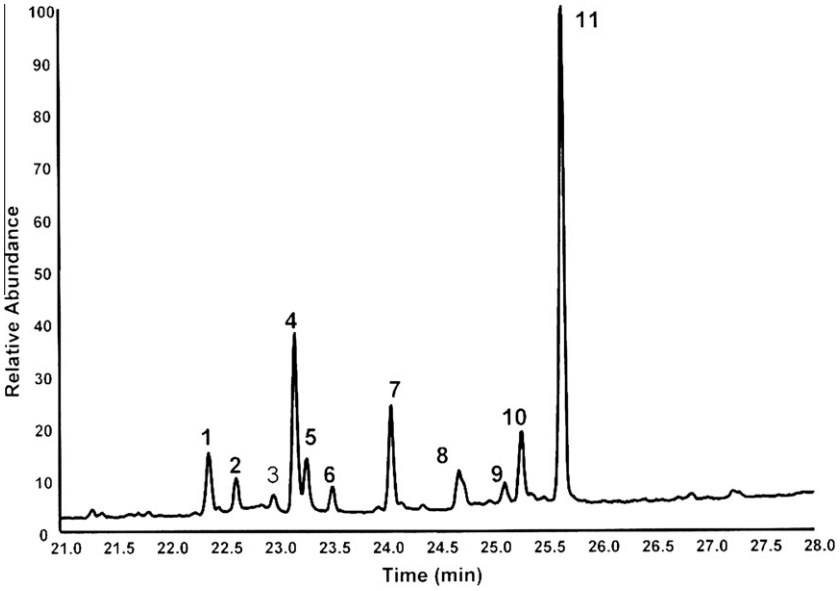

Fig. 2. GC–MS analysis of the culture filtrate of *F. sacchari* strain B-12756. The total ion current is shown for an ethyl acetate extract after derivatization to methyl esters and trimethylsilyl ethers. Main components, identified by comparison of their mass spectra with published data (Gaskin and MacMillan, 1992), are indicated: peak 1, GA_{14} (**12**); peak 2, GA_4 (**15**); peak 3, GA_7 (**17**); peak 4, fujenoic acid (**7**); peak 5, fujenoic triacid (**8**); peak 6, gibberellenic acid; peak 7, GA_{13} (**16**); peak 8, GA_{47} (**25**)/iso-GA_3/GA_{16} (**23**); peak 9; 7β,18-dihydroxykaurenolide (**5**); peak 10, GA_1(**18**); peak 11, GA_3 (**19**).

(**23**) (1α-hydroxyGA_4) and 2-epi-GA_{43} (2α-hydroxyGA_{13}) may be by-products of the 1,2-desaturase (Table 1, Fig. 3).

The amounts of GA_3 (**19**) present in cultures of the most active *F. sacchari* strains B-12756 and B-1732 were lower than those found in *F. fujikuroi* IMI 58289 cultures, which accumulated about 109.2 μg/mL of GA_3 (**19**). In an attempt to increase GA biosynthesis, strain B-12756 was complemented with plasmid pGKScos1, carrying the *F. fujikuroi ggs2* and *cps-ks* genes. The reactions catalyzed by GGS2 and CPS-KS have been demonstrated to be blocked in *F. proliferatum* non-producing strains (Malonek et al., 2005b,c) and could be limiting in *F. sacchari*. The transformants were examined by Southern blot analysis to show if additional copies of *ggs2* and *cps-ks* were correctly inserted into the genome. Five of the 24 transformants, T1, T2, T3, T7 and T11, were shown to carry additional gene copies from the *F. fujikuroi* genome and produced significantly increased amounts of GA_3 (**19**) after 10 days growth in 10% ICI medium (Fig. 4, Table 2). Particularly the cultures of transformants T2 and T3 contained 78.6 and 83.2 μg/mL of GA_3 (**19**), values that are respectively 2.8 and 2.9 times higher than that in B-12756. This indicates that the early steps of the pathway, GGDP synthesis and/or cyclization of this intermediate into *ent*-kaurene (**3**), are limiting reactions in GA biosynthesis by *F. sacchari*.

Table 2
GA_3 (**19**) levels in *F. sacchari* (MP-B) cultures. Effect of complementation with *Ffggs2* and *Ffcps-ks*.

Strain	[GA_3] (**19**) (μg/ml)	Relative GA_3 (**19**) levels
F. sacchari B-12756	28.4	1
F. sacchari B-1732	27.21	0.96
F. sacchari B-7610	11.36	0.4
F. sacchari B-1721	2.76	0.097
B-12756 T1[a]	53.6	1.89
B-12756 T2[a]	78.6	2.77
B-12756 T3[a]	83.2	2.9
B-12756 T7[a]	68.2	2.4
B-12756 T11[a]	30.6	1.08

[a] Transformants of strain B-12756 complemented with *ggs2* and *cps-ks* genes from *F. fujikuroi*.

2.2. GA oxidase activities in *F. sacchari* strains

The activities of the monooxygenases P450-1 (GA_{14} synthase), P450-2 (GA 20-oxidase) and P450-3 (GA 13-hydroxylase) were investigated in *F. sacchari* cultures by adding ^{14}C-labelled substrates to GA-producing (B-12756, B-1797) and GA-non-producing (B-3828 and B-1725) strains. [$^{14}C_4$]GA_{12}-aldehyde (**9**), a substrate of GA_{14} synthase, was converted by B-12756 and B-1797 into the 3β-hydroxylated C_{19}-GAs (Table 3) [$^{14}C_4$]GA_4 (**15**) (22–33%), [$^{14}C_4$]GA_7 (**17**) (9%) [$^{14}C_4$]GA_3 (**19**) (22–31%) and [$^{14}C_4$]GA_1 (**18**) (6–9%) (Table 3). [$^{14}C_4$]GA_{14} (**12**), an early C_{20}-GA intermediate of the GA pathway also accumulated in the culture fluids (16–23%). In addition the extracts from [$^{14}C_4$]GA_{12}-aldehyde (**9**) contained low levels of [$^{14}C_4$]GA_{13} (**16**), a by-product of 20-oxidase in which the methyl group is oxidized to a carboxylic acid (Tudzynski et al., 2002). Non-hydroxylated ^{14}C-GAs were not formed from [$^{14}C_4$]GA_{12}-aldehyde (**9**). The GA 20-oxidase activity (P450-2) was directly assayed in strain B-12756 with the non-hydroxylated substrate [$^{14}C_1$]GA_{12} (**11**), which was converted into the final oxidation products [$^{14}C_1$]GA_9 (**14**) and [$^{14}C_1$]GA_{25} (**13**) plus significant amounts of the C-20 alcohol and C-20 oxo intermediates, [$^{14}C_1$]GA_{15} (**20**) and [$^{14}C_1$]GA_{24} (**21**) (Table 3, Fig. 3). This differs from [$^{14}C_1$]GA_{12} (**11**) oxidation by the *F. fujikuroi* 20-oxidase, that gives only final oxidation products without accumulation of partially oxidized intermediates (Tudzynski et al., 2002), and indicates a lower 20-oxidase activity in *F. sacchari*. Finally, 13-hydroxylase (P450-3) was assayed in B-12756 and B-1797 strains with [$^{14}C_1$]GA_7 (**17**) and [$^{14}C_1$]GA_9 (**14**) as substrates. [$^{14}C_1$]GA_7 (**17**) was converted into [$^{14}C_1$]GA_3 (25–27%) while the non-hydroxylated substrate [$^{14}C_1$]GA_9 (**14**) was not utilized by this monooxygenase but was rather converted into [$^{14}C_1$]GA_{10} (16,17-hydrated-GA_9) plus [$^{14}C_1$]GA_{40} (**24**) (2α-OH-GA_9). GA_{10} would be generated non-enzymatically or non-specifically by enzymes of other pathways, while GA_{40} (**24**) would be formed as a by-product by the GA desaturase (Tudzynski et al., 2003). The low amount of [$^{14}C_4$]GA_1 (**18**) found in incubations with [$^{14}C_4$]GA_{12} aldehyde (**9**) in which [$^{14}C_4$]GA_4 (**15**) accumulates suggests that the *F. sacchari* 13-hydroxylase utilizes GA_4 (**15**) with low efficiency. On the other hand, the GA desaturase was found to oxidize several GA

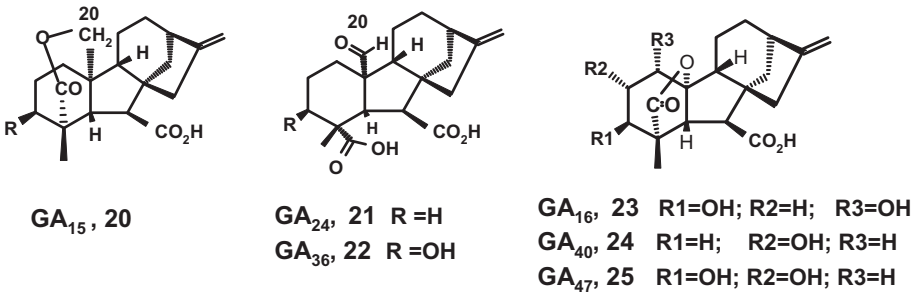

Fig. 3. Structures of GAs formed in *F. sacchari* and *F. konzum* cultures.

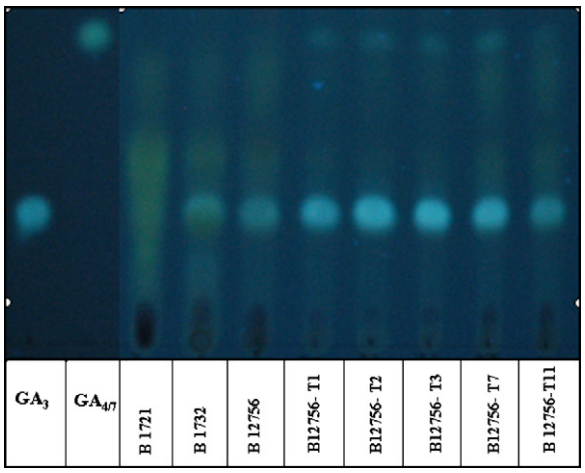

Fig. 4. TLC analysis of GA_3 (**19**) in culture filtrates of *F. sacchari* wild-type strains (B-1721, B-1732, B-12756) and B-12756 transformants with *ggs2* and *cps-ks* genes from *F. fujikuroi* (T1, T2, T3, T7, T11) after 10 days of cultivation in 10% ICI medium.

substrates, GA_4 (**15**), GA_9 (**14**) and GA_{13} (**16**). GA_4 (**15**) was mainly converted to the 1,2-unsaturated product GA_7 (**17**) (Table 3) plus the 1α-hydroxy and 2α-hydroxy derivatives, GA_{16} (**23**) and GA_{47} (**25**) (Fig. 3) that were detected as endogenous metabolites in B-12756 and B-1797 cultures (Table 1). The 2α-hydroxy derivatives of GA_9 (**14**) and GA_{13} (**16**), GA_{40} (**24**) and GA_{43} (2α-hydroxyGA_{13}), respectively, are also likely to be by-products of the GA desaturase (Tables 1 and 3, Fig. 3).

In contrast to B-12756 and B-1797, GA oxidase activities were not detectable in strains B-3828 or B-1725, which did not convert ^{14}C-labelled substrates into either intermediates or final products of the GA pathway. [$^{14}C_1$]GA_7 (**17**) and [$^{14}C_1$]GA_9 (**14**) were recovered unconverted while 70% of [$^{14}C_4$]GA_{12} aldehyde (**9**) was recovered, with the remainder converted to products with HPLC retention times that did not correspond to any products of the GA pathway and are assumed to be due to non-specific metabolism. Probably low expression of the GA genes, as described for *cps-ks, P450-4* and *des* in strain B-00278 (Malonek et al., 2005a), would account for the GA oxidase activities not being detected in these *F. sacchari* isolates.

2.3. Gibberellin biosynthesis in F. konzum (MP-I) isolates

Six *F. konzum* isolates were tested for GA biosynthesis by GC–MS analysis of EtOAc extracts after 10 days of incubation in liquid media containing limiting nitrogen (Table 4). As with *F. sacchari*, GA-producing and GA-non-producing *F. konzum* strains were found. Besides I-10653 that had been previously described to produce GAs (Malonek et al., 2005a), the cultures of two other strains (I-11616 and I-11893) were found to contain GAs. GA_1 (**18**) was the main final product while GA_3 (**19**) accumulated at half of the level of GA_1(**18**), in contrast to *F. sacchari* isolates that synthesized mainly GA_3 (**19**) (Tables 1 and 4). GA_1 (**18**) levels were low for all the *F. konzum* isolates (less than 0.1 μg/mL), much lower than the levels found in active *F. sacchari* strains. No intermediates of GA biosynthesis were detected in I-11616 and I-11893, while the cultures of I-10653 contained 3β-hydroxylated intermediates and by-products, GA_4 (**15**), GA_7 (**17**), GA_{13} (**16**), GA_{47} (**25**) and GA_{16} (**23**), besides GA_1 (**18**) and GA_3 (**19**) (Table 4). In contrast, the cultures of strains I-10578, I-10595 and I-10678 contained no GAs or *ent*-kaurenoids, except for traces of GA_1 (**18**) found in I-10578. These results confirm the existence of GA producing and non-producing strains in *F. konzum* and demonstrate inefficient GA biosynthesis in the active strains.

2.4. Utilization of ^{14}C-labelled GA precursors by F. konzum I-10653 (GA-producing strain) and I-10595 (GA-non-producing strain)

Cultures of I-10653 completely oxidized [$^{14}C_4$]GA_{12}-aldehyde (**9**) into [$^{14}C_4$]GA_4 (**15**) and [$^{14}C_4$]GA_7 (**17**), as well as a number of unidentified products, which from their mass spectra would appear to be C_{20}-GAs (Table 5). The mass spectra of the more abundant of these products are consistent with them being mono- and dihydroxylated tricarboxylic acids as well as a monounsaturated form, but they did not correspond to any of the known GAs. No [$^{14}C_4$]GA_1 (**18**) or [$^{14}C_4$]GA_3 (**19**) were formed from this precursor, suggesting relatively low desaturase and 13-hydroxylase activities. This was confirmed by the only partial conversion of [$^{14}C_1$]GA_4 (**15**) into 13-hydroxylated products [$^{14}C_1$]GA_1 (**18**) (5%) and [$^{14}C_1$]GA_3 (**19**) (25%) with some accumulation of the unsaturated intermediate [$^{14}C_1$]GA_7 (**17**) (13%). The small amount of [$^{14}C_1$]GA_1 (**18**) relative to [$^{14}C_1$]GA_3 (**19**) may result from a higher level of substrate available for the desaturase in [$^{14}C_1$]GA_4 (**15**) incubations compared to endogenous conditions. The non-hydroxylated substrate [$^{14}C_1$]GA_9 (**14**) was partially converted (20%) to an unknown more polar product that could not be detected by GC–MS.

Additionally 20-oxidation was assayed with [$^{14}C_1$]GA_{12} (**11**), which was transformed by I-10653 into the partially oxidized 20-alcohol and 20-oxo products, [$^{14}C_1$]GA_{15} (**20**) and [$^{14}C_1$]GA_{24} (**21**), respectively, besides the final products of C-20 oxidation, [$^{14}C_1$]GA_9 (**14**) and [$^{14}C_1$]GA_{25} (**13**). C_{20}-GA intermediates of the 3β-hydroxylation pathway did not accumulate indicating that 20-oxidation was less efficient with the non-hydroxylated substrates than with the 3β-hydroxylated intermediates. It has been described for *F. fujikuroi* that the 20-oxidase utilizes both GA_{14} (**12**) and GA_{12} (**11**) without accumulation of intermediates (Tudzynski et al., 2002). Probably the high enzymatic activity present in *F. fujikuroi* cultures that gives complete conversion of both substrates does not allow intermediates to accumulate for either pathway.

Table 3
Utilization of ^{14}C-GA precursors by cultures of strains B-12756 and B-1797.

Strain	Substrate	Products[a] (%)	Mass spectrum *m/z* (% relative abundance)
B-12756	[$^{14}C_4$]GA_{12}-aldehyde, **9**	[$^{14}C_4$]GA_3, **19** (31%)	512(18), 510(8), 504(100), 489(11), 475(6), 460(7), 445(10), 370(15), 347(14), 311(8), 238(12), 208(41)
		[$^{14}C_4$]GA_1, **18** (6%)	514(25), 512(15), 506(100), 491(11), 448(18), 377(11), 376(12), 357(4), 313(4), 209(10), 207(17)
		[$^{14}C_4$]GA_4, **15** (22%)	426(16), 424(9), 418(21), 394(17), 390(17), 386(20), 336(20), 328(26), 295(41), 292(62), 290(67), 289(67), 284(100), 233(52), 231(59), 230(54), 229(61), 225(90), 224(62), 131(35), 129(46)
		[$^{14}C_4$]GA_{42} (6%)	546(4), 531(26), 529(14), 523(16), 488(22), 481(11), 456(10), 448(11), 424(18), 416(22), 384(100), 382(60), 376(68), 322(29), 293(53), 287(33), 263(30), 235(48), 131(56)
		[$^{14}C_4$]GA_7, **17** (9%)	424(20), 416(28), 288(20), 282(37), 229(40), 228(67), 223(63), 222(100)
		[$^{14}C_4$]GA_{14} **12** (23%)	456(5), 441(21), 424(61), 416(31), 396(55), 388(21), 334(36), 306(76), 304(54), 298(43), 293(97), 291(86), 287(53), 263(74), 245(54), 235(100), 231(32), 131(84)
		[$^{14}C_4$]GA_{13}, **16** (3%)	477(25), 460(30), 436(38), 408(15), 400(77), 372(18), 349(33), 342(25), 318(13), 310(66), 283(41), 282(50), 251(18), 223(125), 131(38), 129(100)
	[$^{14}C_1$]GA_{12}, **11**	[$^{14}C_1$]GA_{15}, **20** (9%)	346(15), 314(16), 300(11), 286(42), 243(22), 241(100), 195(16)
		[$^{14}C_1$]GA_{24}, **21** (55%)	376(3), 348(7), 344(35), 316(98), 312(35), 300(9), 298(9), 288(85), 287(63), 285(33), 284(38), 257(23), 256(67), 239(12), 229(67), 228(97), 227(100)
		[$^{14}C_1$]GA_{25}, **13** (9%)	374(26), 314(74), 286(100), 227(52), 226(13)
		[$^{14}C_1$]GA_9, **14** (27%)	332(8), 300(100), 288(15), 272(95), 243(54), 229(56), 228(59), 217(27), 159(32)
	[$^{14}C_1$]GA_7, **17**	[$^{14}C_1$]GA_3, **19** (27%)	506(44), 504(100), 489(7), 475(5), 460(4), 445(7), 372(11), 370(26), 355(11), 347(8), 311(10), 238(11), 208(20)
	[$^{14}C_1$]GA_9, **14**	[$^{14}C_1$]GA_{40}, **24** (24%)	420(1), 405(3), 389(5), 373(100), 345(95), 329(5), 327(6), 301(87), 286(63), 271(8), 245(6), 227(49), 225(34)
		[$^{14}C_1$]GA_{10} (6%)	422(91), 407(22), 405(44), 391(9), 378(20), 363(50), 349(10), 347(18), 331(100), 303(24), 288(21), 273(26), 245(31), 227(28), 132(98)
B-1797	[$^{14}C_4$]GA_{12}-aldehyde[b], **9**	[$^{14}C_4$]GA_3, **19** (22%)	512(30), 510(16), 506(15), 504(100), 489(9), 475(3), 460(6), 445(9), 370(9), 347(16), 311(11), 238(9), 210(18), 208(34)
		[$^{14}C_4$]GA_1, **18** (9%)	514(80), 512(40), 506(100), 499(6), 497(5), 491(11), 455(12), 454(11), 448(12), 381(13), 377(11), 376(11), 319(8), 317(9), 209(16), 207(13)
		[$^{14}C_4$]GA_{13}, **16** (trace)	485(9), 477(22), 442(13), 436(24), 408(33), 400(54), 378(13), 372(18), 318(30), 310(49), 288(22), 282(40), 229(19), 223(12), 131(63), 129(100)
		[$^{14}C_4$]GA_4, **15** (33%)	425(23), 418(17), 394(25), 386(17), 336(27), 328(18), 295(69), 292(100), 290(71), 289(56), 284(74), 237(50), 233(51), 231(83), 230(84), 229(79), 225(69), 224(53), 131(56), 129(49)
		[$^{14}C_4$]GA_{14}, **12** (16%)	456(3), 441(17), 424(61), 416(25), 396(47), 388(22), 334(33), 306(80), 304(57), 298(35), 293(97), 291(74), 287(56), 263(74), 245(56), 235(100), 231(47), 131(80)
		[$^{14}C_4$]GA_7, **17** (trace)	424(13), 416(17), 288(29), 282(20), 229(56), 228(100), 223(71), 222(96)
	[$^{14}C_1$]GA_7, **17**	[$^{14}C_1$]GA_3, **19** (25%)	506(100), 504(98), 491(9), 489(9), 447(9), 445(10), 389(8), 387(10), 372(11), 370(11), 349(14), 347(14), 223(9), 221(9), 210(34), 208(36)
		[$^{14}C_1$]GA_7, **17** (75%)	Not analyzed
	[$^{14}C_1$]GA_9, **14**	[$^{14}C_1$]GA_{40}, **24** (7%)	405(7), 373(100), 345(95), 317(9), 301(83), 286(63), 271(11), 245(8), 227(50), 225(39)
		[$^{14}C_1$]GA_{10} (13%)	422(93), 407(24), 405(49), 378(22), 363(51), 347(22), 331(99), 303(26), 288(21), 273(23), 245(35), 227(29), 132(100)
		[$^{14}C_1$]GA_9, **14** (80%)	Not analyzed

[a] Identified in HPLC fractions of EtOAc extracts of the culture fluid.
[b] Unconverted substrate (20%) was recovered in the mycelial methanol extract.

Table 4
GAs present in the culture fluid from *F. konzum* (MP-I) strains.

Strain	Gibberellins	GA_1:GA_3
I-10653	GA_4, **15**; GA_7, **17**; GA_{13}, **16**; GA_{47}, **25**; iso-GA_3; GA_{16}, **23**; GA_1, **18**; GA_3, **19**	2:1
I-11616	GA_1, **18**; GA_3, **19**	2:1
I-11893	GA_1, **18**; GA_3, **19**	2:1
I-10578	Trace GA_1, **18**	No GA_3 detected
I-10595	No GAs detected	No GAs detected
I-10678	No GAs detected	No GAs detected

Altogether these results suggest that the GA oxidases have a low activity in *F. konzum* strain I-10653 compared to *F. fujikuroi*. This was confirmed by measuring the rates of oxidation of the substrates for GA_{14} synthase and GA 20-oxidase in liquid cultures of *F. konzum* I-10653 and *F. fujikuroi* IMI58289 (Fig. 5, exemplified for 20-oxidase). Equal dry weight of mycelia (10 mg/mL) was used in each experiment, *ent*-[$^{14}C_1$]kaurenoic acid (**4**) or [$^{14}C_1$]GA_{12} (**11**) was added to the cultures and the remaining substrate was quantified at different times by HPLC. Fifty percent of [$^{14}C_1$]GA_{12} (**11**), a substrate for the 20-oxidase, was oxidized in 9 min by IMI58289 whereas I-10653 cultures converted 50% of this substrate in 37 h (Fig. 5). Similarly, while IMI58289 oxidized 50% of *ent*-[$^{14}C_1$]kaurenoic acid (**4**) to [$^{14}C_1$]GAs in 30 min, I-10653 required 72 h to achieve this level of conversion.

In contrast to strain I-10653, the GA-non-producing strain I-10595 did not give any products of the GA pathway when incubated with the GA precursors *ent*-7α-hydroxy[$^{14}C_4$]kaurenoic acid (**6**), [$^{14}C_4$]GA_{12}-aldehyde (**9**), [$^{14}C_1$]GA_{14} (**12**) or [$^{14}C_1$]GA_4 (**15**). Besides unconverted substrate, hydrated *ent*-7α-hydroxy[$^{14}C_4$]kaurenoic acid was identified by GC–MS in incubations with *ent*-7α-hydroxy[$^{14}C_4$]kaurenoic acid (**6**) while [$^{14}C_1$]GA_{14} (**12**) was partially converted to a [$^{14}C_1$]GA_{14} isomer probably formed non-enzymatically during extraction. The only GA-biosynthetic activity detected was a low 20-oxidase activity that gave 15% conversion of [$^{14}C_1$]GA_{12} (**11**) into hydrated [$^{14}C_1$]GA_9.

2.5. Absence of GA oxidase activities in F. subglutinans strains E-2192 and E-00990

The two *F. subglutinans* strains tested, E-2192 and E-00990, contained no GAs or *ent*-kaurenoids in extracts of the culture fluids obtained from 10 days incubation in nitrogen-limiting medium, when analyzed by GC–MS. Neither strain showed activity for any of the GA oxidases when assayed with ^{14}C-labelled substrates. *ent*-[$^{14}C_4$]Kaurene (**3**) was recovered unconverted in the mycelial extract after 3 days of incubation while the filtrate extracts from incubations of *ent*-7α-hydroxy[$^{14}C_4$]kaurenoic acid (**6**) and [$^{14}C_4$]GA_{12}-aldehyde (**9**) gave no detectable products by GC–MS. The substrates of the GA 20-oxidase, [$^{14}C_1$]GA_{12} (**11**) and [$^{14}C_1$]GA_{14} (**12**), were transformed by *F. subglutinans* strains into [$^{14}C_1$]GA_{12} hydrate (12%) and [$^{14}C_1$]GA_{14} hydrate (15%), respectively, while [$^{14}C_1$]GA_4 (**15**) and [$^{14}C_1$]GA_7 (**17**) were partially transformed into [$^{14}C_1$]GA_4 3-acetate (30%) plus [$^{14}C_1$]GA_2 (6%) and to iso[$^{14}C_1$]GA_7 (30%), respectively, i.e. all reactions that are unrelated to GA biosynthesis.

3. Discussion

Within the *G. fujikuroi* species complex, the ability to synthesize GAs has been mainly described for the rice pathogen *F. fujikuroi*, a member of the Asian clade that produces gibberellic acid (GA_3, **19**). Here we have characterized two additional *Fusarium* species of the complex that have some GA-producing strains, as well as others that do not synthesize GAs: *F. sacchari* isolated from sugar cane or sorghum fields in Asia (Leslie, 1995) and *F. konzum* isolated from prairie grasses in America (USA) (Zeller et al., 2003). Inactive strains of *F. sacchari* have been previously described (Malonek et al., 2005a). We have now extended the list of *F. sacchari* strains tested and found that some are active in GA biosynthesis while others synthesize only traces of GAs and do not metabolize externally added ^{14}C-labelled GA oxidase substrates. The GA-containing *F. sacchari* strains accumulated significant amounts of GA_3 (**19**), although lower than those found in cultures of the *F. fujikuroi* strain IMI 58289. The levels of GA_3 (**19**) in the cultures were increased 2.9 times by complementing strain B-12756 with *ggs2* and *cps-ks* from *F. fujikuroi* which indicates that at least one of these is limiting for GA biosynthesis in this strain. *F. sacchari* belongs to the Asian clade of the *G. fujikuroi* complex and is closely related to *F. fujikuroi* which suggests that GA biosynthesis may be mainly associated with members of this group in the complex. For *F. proliferatum*, another species from the Asian clade, two GA-producing strains have been described (Rim et al., 2005; Tsavkelova et al., 2008) in contrast to other *F. proliferatum* strains that do not produce GAs (Malonek et al., 2005a). The *F. proliferatum* isolate ET1 obtained from the roots of an orchid gives low levels of GA_3 (**19**), but relatively high levels of the precursors GA_7 (**17**) and GA_4 (**15**) (Tsavkelova et al., 2008), while *F. proliferatum* KGL0401 produce high amounts of GA_3 (**19**) and other GAs (Rim et al., 2005). Interestingly, the *F. proliferatum* GA-producing strains are endophytes that have been isolated from roots while the non-producing strains were plant pathogens in maize.

GA biosynthesis has also been suggested to occur in *F. mangiferae*, a fungus that causes mango malformation disease, resulting in important yield losses (Leslie and Summerell, 2006; Marasas et al., 2006). Interestingly, *F. mangiferae* is phylogenetically closely related to *F. sacchari* and to *F. fujikuroi* and belongs to the Asian clade. Its effect has been attributed to production of GAs and/or cytokinins although this has not been yet demonstrated (Leslie and Summerell, 2006).

Besides *F. sacchari*, we found that some strains of *F. konzum*, a species distantly related to *F. fujikuroi* that belongs to the American clade, can synthesize GAs. Three of the tested strains, I-10653, I-11616 and I-11893, produce mainly GA_1 (**18**) in contrast to *F. sacchari* isolates that gave mainly GA_3 (**19**) as final product. Another difference is the very low level of GAs found in the three *F. konzum* active strains (less than 0.1 μg/mL). Reduced activities of the GA oxidases were demonstrated in I-10653 compared to *F. fujikuroi*

Table 5
Products from incubations of ^{14}C-GA precursors with *F. konzum* strain I-10653.

Substrate	Products (%)	Mass spectrum m/z (%relative abundance)
[$^{14}C_4$]GA_{12}-aldehyde, **9**	[$^{14}C_4$]GA_4, **15** (26%)	426(30), 424(14), 418(11), 394(31), 364(16), 336(36), 295(70), 292(100), 290(74), 284(38), 237(50), 233(47), 231(82), 230(80), 229(56), 225(27), 224(26), 131(42)
	[$^{14}C_4$]GA_7, **17** (20%)	424(23), 422(13), 416(7), 396(11), 290(35), 288(21), 282(12), 229(72), 228(100), 227(44), 226(53), 223(35), 222(39), 197(26)
	Unknown 1 [M^+ 420] (4%)	428(63), 426(37), 420(14), 400(49), 392(16), 318(51), 310(28), 297(100), 295(66), 291(56), 269(70), 267(36), 263(26), 239(71), 217(70), 131(81), 129(52)
	Unknown 2 [M^+ 492] (24%)	500(100), 498(60), 492(32), 485(10), 477(3), 369(13), 366(32), 364(12), 358(5), 340(19), 338(14), 332(9), 305(14), 299(5), 279(8), 275(6)
	Unknown 3 [M^+ 490] (3%) – weak spectrum	498(20), 496(12), 490(6), 453(22), 400(34), 338(34), 131(100), 129(29)
	Unknown 4 [M^+ 490] (11%)	498(100), 496(53), 490(35), 483(11), 332(13), 303(35), 259(58), 228(40)
	Unknown 5 [M^+ 580] (12%)	588(100), 586(55), 580(28), 573(26)
[$^{14}C_1$]GA_{12}, **11**	[$^{14}C_1$]GA_{15}, **20** (12%)	346(12), 314(28), 300(23), 286(78), 268(9), 241(100), 240(41), 195(49)
	[$^{14}C_1$]GA_{24}, **21** (35%)	376(1), 348(5), 344(17), 316(66), 312(83), 300(16), 298(23), 288(65), 285(41), 284(42), 256(67), 243(12), 239(14), 229(44), 228(100), 227(84)
	[$^{14}C_1$]GA_{25}, **13** (5%)	374(15), 314(76), 286(100), 227(35), 226(31)
	[$^{14}C_1$]GA_9, **14** (35%)	332(2), 300(38), 288(10), 272(100), 254(8), 243(47), 228(45), 226(34), 213(14), 185(16), 171(16)
[$^{14}C_1$]GA_4, **15**	[$^{14}C_1$]GA_3, **19** (25%)	506(97), 504(100), 491(7), 489(7), 447(7), 445(8), 389(5), 387(8), 372(9), 370(9), 349(10), 347(11), 299(6), 297(8), 210(22), 208(30)
	[$^{14}C_1$]GA_1, **18** (5%)	508(98), 506(100), 493(10), 491(10), 450(20), 448(20), 377(18), 376(15), 315(9), 313(10), 209(22), 207(19)
	Gibberellenic acid (5%)	520(25), 518(31), 460(36), 458(44), 410(59), 399(65), 371(100), 369(91), 311(41), 309(48)
	GA_{16}, **23** + GA_{47}, **25** (2%)	Mixed spectrum
	[$^{14}C_1$]GA_7, **17** (13%)	418(19), 416(20), 386(11), 384(10), 358(12), 356(11), 300(20), 298(14), 284(31), 282(40), 224(100), 222(91), 195(23), 193(25)
	[$^{14}C_1$]GA_4, **15** (50%)	420(20), 418(17), 392(12), 390(15), 388(22), 386(18), 360(13), 358(12), 330(29), 328(20), 291(49), 289(44), 286(100), 284(94), 263(24), 261(21), 227(75), 226(79), 225(80), 226(64), 129(47)
[$^{14}C_1$]GA_9, **14**	[$^{14}C_1$]GA_9, **14** (80%)[a]	332(1), 300(41), 288(10), 272(100), 254(8), 243(44), 228(49), 226(35), 213(13), 185(16), 171(16)

[a] Twenty percent of the substrate was converted into an unknown more polar product which could not be detected by GC–MS.

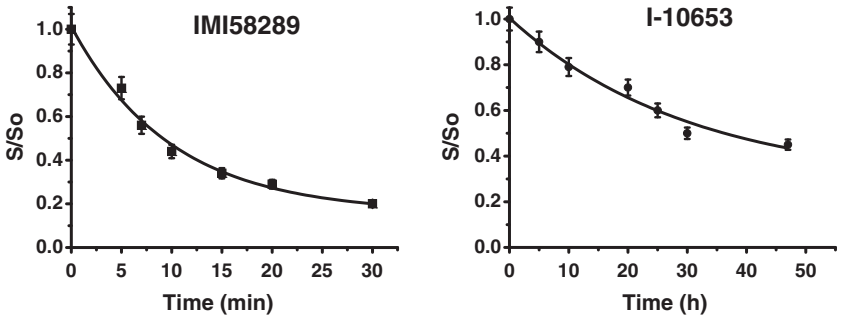

Fig. 5. Time course of [$^{14}C_1$]GA_{12} (**11**) oxidation by cultures of *F. konzum* strain I-10653 and *F. fujikuroi* strain IMI 58289. Equal amounts of mycelia (10 mg d.w./mL) were incubated with the substrate in 0% ICI medium and the remaining substrate was quantified by HPLC. S/So is the ratio between remaining substrate and initial substrate concentration. Values shown are the result of three independent experiments. Error bars indicate standard deviations.

IMI 58289, which could be due to low expression of the GA biosynthesis genes and/or to mutations in the coding region, as found for *F. proliferatum* (Malonek et al., 2005b, c). Expression of *des* and *cps-ks* has been described in I-10653 although at a lower level than in *F. fujikuroi* strains, in contrast to the GA-non-producing strain I-10595 that showed no expression of these genes (Malonek et al., 2005a). In agreement with this, we found no GA oxidase activities in the cultures of I-10595. For *F. subglutinans*, another member of the American clade studied, the two strains tested did not synthesize GAs or *ent*-kaurenoids and contained no activity of the GA oxidases, in agreement with the absence of expression of the GA genes reported for these strains (Malonek et al., 2005a).

The fact that *F. konzum, F. sacchari* and *F. proliferatum* isolates contain GA-producing, as well as GA-non-producing strains, would indicate that these species are in the process of losing their abilities to synthesize these diterpene phytohormones. Variability between strains has also been described for the biosynthesis of several mycotoxins by *F. konzum* and *F. proliferatum* (Leslie et al., 2004). As for GA biosynthesis, *F. konzum* isolates can be separated into producers and non-producers of fumonisins and fusaproliferin while *F. proliferatum* contain strains that produce fumonisins, fusaproliferin and beauvericin and others that do not synthesize these mycotoxins (Leslie et al., 2004). The *F. konzum* non-GA-producing strains have no activity for any of the GA oxidases as found for *F. sacchari* inactive strains B-3828 and B-1725 that did not convert ^{14}C-labelled precursors into [^{14}C]GAs. Thus, within the *G. fujikuroi* species complex, GA biosynthesis would be mainly present in the members of the Asian clade: *F. fujikuroi*, *F. sacchari* and *F. proliferatum*, all closely related species.

The ability of different *F. sacchari* and *F. konzum* strains to synthesize GAs could be related to the type of interaction they have with their host plants, as found for *F. proliferatum*. Endophytes of *F. proliferatum* isolated from either orchid roots or from the roots of *Physalis alkekengi* are able to produce GAs while pathogenic strains of the same species, isolated from maize, do not have this ability, although all *F. proliferatum* strains contain the entire GA biosynthesis gene cluster (Malonek et al., 2005a; Tsavkelova et al., 2008; Rim et al., 2005). *F. proliferatum* ET1 is also able to produce significant amounts of auxins in contrast to other *F. proliferatum* strains, suggesting that phytohormone production might be important for an endophytic life style (Tsavkelova et al., 2008). Interestingly, for the rice pathogen *F. fujikuroi*, it has been suggested that secreted GAs would be a virulence factor since GAs have been found to interfere with jasmonate signaling, which forms part of the mechanism for plant resistance to necrotrophic fungi such as *F. fujikuroi* (Navarro et al., 2008). The examination of several isolates for each species of the *G. fujikuroi* complex for their ability to produce GAs and other plant hormones will help to better understand the possible connection between phytohormone production and life style and/or host-plant interaction.

3.1. Concluding remarks

Most *Fusarium* species within the *G. fujikuroi* complex contain the entire GA biosynthesis gene cluster; however, so far the ability to synthesize GAs was mainly described for *F. fujikuroi,* a rice pathogen that produces high amounts of GA_3. Our results with three other species of the complex demonstrate that GA biosynthesis is also present in some *F. sacchari* and *F. konzum* isolates although they differ in the levels of GAs synthesized and in the final products formed. Significant GA levels were found in *F. sacchari* cultures as previously described for particular *F. proliferatum* isolates, both species phylogenetically closely related to *F. fujikuroi*. In contrast, inefficient GA biosynthesis was demonstrated for *F. konzum* and inactive strains were found for *F. subglutinans*, two distantly related species. These results suggest that the ability to synthesize GAs would be mainly associated to species closely related to *F. fujikuroi*. Further characterization of other *Fusarium* species that contain the GA biosynthesis genes, will give more insight into the distribution of GA biosynthesis within the *G. fujikuroi* complex.

4. Experimental

4.1. Fungal strains and plasmids

F. sacchari isolates B-1721, B-1725, B-1732, B-1797, B-3828 and B-12756 were provided by Dr. J.F. Leslie (Kansas State University, USA). Strain B-7610 was obtained from the Fungal Genetics Stock Center, University of Missouri, Kansas, USA. *F. konzum* strains I-10578, I-10595, I-10653, I-10678, I-11616, I-11893, as well as *F. subglutinans* E-00990 and E-2192 were provided by Dr. J.F. Leslie (Kansas State University, USA). The wild-type *F. fujikuroi* strain IMI 58289 was obtained from Dr. J. Avalos, Universidad de Sevilla, Spain. Plasmid pGKScos1, containing both genes, *ggs2* and *cps-ks* of *F. fujikuroi* strain m567, was constructed by P. Linnemannstöns (unpublished data). Strains of *F. sacchari* complemented with *ggs2* and *cps-ks* from *F. fujikuroi* (accession numbers: Y15013; Y15280) were obtained by transforming protoplasts of strain B-12756 with the vector pGKScos1. To generate this vector, a 6.4 kb *KpnI*/*SacI* genomic fragment carrying the *ggs2* and *cps-ks* genes, were cloned into pUC19 resulting in plasmid pGKS. To generate a vector carrying in addition to these genes a resistant marker, plamid pGPC1 (Desjardins et al., 1992) containing the hygromycin resistance cassette and the *cos* recognition site was linearized by BamHI and combined with BamHI-restricted vector

pGKS (ratio 1:1) by in vitro packaging of a lambda phage (Stratagene, Germany). Phages were used to transfect *E. coli* resulting in vector pGKScos1.

4.2. Transformation of F. sacchari strain B-12756

The preparation of protoplasts and the transformation procedure were as previously described (Malonek et al., 2005b). For complementation experiments, 10^7 protoplasts (50 μl) of strain B-12756 were transformed with 10 μg of the plasmid pGKScos1 containing both the *F. fujikuroi ggs2* and *cps-ks* genes. Transformed protoplasts were regenerated at 28 °C on complete regeneration agar (0.7 M sucrose, 0.05% yeast extract, 0.1% $(NH_4)_2SO_4$) containing 120 μg/ml hygromycin B (Calbiochem, Bad Soden, Germany) for 5–6 days. To confirm the integration of the plasmid pGKScos1 into the genome, transformants were analyzed by Southern blot analysis. The genomic DNA of the wild-type B-12756 and the transformants were isolated and restricted with *BamHI*. The restricted DNA was blotted onto Nylon membranes and hybridized with the 6.4 kb genomic fragment carrying the *ggs2* and *cps-ks* genes of *F. fujikuroi* as probe. Transformants carrying additional hybridizing bands with a size larger than 6.4 kb compared to the wild-type were used for further analysis.

4.3. Media and culture conditions

Fungal cultures were maintained in potato dextrose agar and inoculated into 40% ICI (Imperial Chemical Industries) liquid medium containing 8% glucose, 0.5% $MgSO_4$, 0.1% KH_2PO_4 and 2 g/L ammonium nitrate (Geissmann et al., 1966). After 3–4 days of incubation at 28 °C in an orbital shaker (200 rpm), the mycelia were washed and transferred into nitrogen-limiting ICI medium with the same composition but containing 0.5 g/L ammonium nitrate (10% ICI) or without ammonium nitrate (0%ICI). 10% ICI medium was used for analysis of the GA profile, while 0% ICI medium was utilized for experiments with labelled precursors. 200 μM AMO 1618 (2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidinecarboxylate methyl chloride) was added to the culture in metabolism experiments to inhibit endogenous GA biosynthesis (Rademacher, 1992).

4.4. Labelled substrates

[$^{14}C_4$]GA_{12}-aldehyde (**9**), *ent*-[$^{14}C_4$]kaurene (**3**) and *ent*-7α-hydroxy-[$^{14}C_4$]kaurenoic acid (**6**) were synthesized from *R*-[2-^{14}C]mevalonic acid (Amersham) by incubation with an endosperm preparation from *Cucurbita maxima* in the presence of ATP, $MgCl_2$ and NADPH (Urrutia et al., 2001). *ent*-[$^{14}C_1$]Kaurenoic acid (**4**), [$^{14}C_1$]GA_{12} (**11**), [$^{14}C_1$]GA_4 (**15**), [$^{14}C_1$]GA_7 (**17**) and [$^{14}C_1$]GA_9 (**14**) were obtained from Professor L. Mander (Australian National University, Canberra, Australia).

4.5. Gibberellin analysis

For analysis of GA production, the different fungal strains were cultivated in 25 mL flasks containing 10 mL of 10% ICI medium (Geissmann et al., 1966) for 10 days on a rotatory shaker (200 rpm) at 28 °C. After acidification of the culture filtrate, GAs were extracted by partition with EtOAc, cleaned by solid phase extraction in C_{18} columns (Rojas et al., 2001) and analyzed by gas chromatography–mass spectrometry (GC–MS) as described in 4.8. GA_3 (**19**) levels were quantified by HPLC in EtOAc extracts of the culture fluid (Barendse et al., 1980).

4.6. Incubation of fungal cultures with ^{14}C-labelled GA precursors

The mycelia grown in 40% ICI were washed and resuspended in 0% ICI medium (Geissmann et al., 1966). One mL aliquots were inoculated into 5 mL of fresh 0% ICI medium in 25 mL flasks and labelled substrates were added as methanol solutions (30,000–500,000 dpm; 0.065–1.85 nmol per flask). The cultures were further incubated for 3 days at 28 °C with shaking at 200 rpm and the products were extracted from the culture filtrate. Partition in EtOAc and solid phase extraction were utilized to purify the products (Rojas et al., 2001) which were further separated by HPLC. For time course experiments mycelia (10 mg d.w./mL) were incubated with labelled substrates at 28 °C in a total volume of 5 mL O% ICI medium. Aliquots were taken at different times and analyzed by HPLC after extraction into EtOAc.

4.7. HPLC conditions

^{14}C-labelled products were separated on a C_{18} Symmetry column (5 μm; 250 × 4 mm; Waters) in a Waters 600 HPLC instrument. A linear gradient from 60% to 100% MeOH in H_2O, pH 3.0, over 30 min was used for elution. The flow rate was 1 mL/min. Fractions were collected and the radioactivity measured by liquid scintillation counting. The GA_3 (**19**) levels in the culture fluids of the different strains were quantified as described (Barendse et al., 1980) in a Merck HPLC system with a UV detector and a Lichrospher 100 RP18 column (5 μm; 250 × 4 mm).

4.8. Product identification

The ^{14}C-labelled products eluted from HPLC were derivatized as methyl esters (ethereal diazomethane), TMSi ethers (N-methyl-N-trimethylsilyltrifluoroacetamide) and identified by GC–MS as described (Troncoso et al., 2008). Compounds were identified by comparison of their mass spectra with those of authentic samples and/or with published spectra (Gaskin and MacMillan, 1992). For endogenous GA determination, the EtOAc extract of the culture filtrate, cleaned by solid phase extraction, was derivatized directly for GC–MS analysis.

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